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Award Number: W81XWH-12-1-0209

TITLE: Three-dimensional ovarian and oviductal culture to enhance transgenic animal studies of cancer and prevention

PRINCIPAL INVESTIGATOR: Joanna E. Burdette

CONTRACTING ORGANIZATION: University of Illinois
809 S. Marshfield, Rm 520
Chicago, IL 60612-4305

REPORT DATE: October-2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30September2012-29September2013

4. TITLE AND SUBTITLE
Three-dimensional ovarian and oviductal culture to enhance transgenic animal studies of cancer and prevention

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0209

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Joanna E. Burdette
Sharon L. Eddie
Suzanne M. Quartuccio
E-Mail: joannab@uic.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES

University of Illinois at Chicago
809 S. Marshfield Avenue, Rm 520
Chicago, IL 60612-4305

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our results confirm that single alterations in pathways associated with high-grade serous cancer are not sufficient to drive soft agar colony formation as an index of transformation, but that specific combinations such as KRAS/PTEN and mutant p53/PTEN, which represent some of the most common pathways activated in human high grade serous cancer, are able to promote early events in tumor formation. Mutation of p53 in the OSE or in the TEC is not sufficient to drive tumorogenesis; however, mutation of p53 combined with PTEN deletion enhances migration and soft agar colony formation. Similarly, PTEN silencing combined with KRAS mutation can enhance oviductal migration and enhance growth in soft agar. These data help support mounting evidence that oviductal epithelium can be a source of serous cancer and provide excellent models for future studies that are aimed at uncovering signaling events downstream of mutant p53, PTEN, KRAS, Rb, BRCA, and Akt.

15. SUBJECT TERMS
ovarian cancer, fallopian tube/oviductal epithelial cells, ovarian surface epithelial cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

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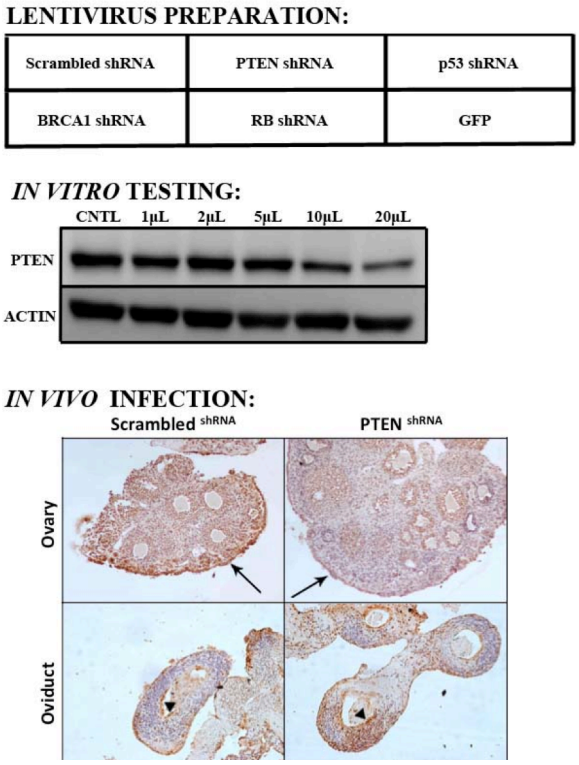
INTRODUCTION: The goal of this grant proposal is to create a culture system for the study of early events in ovarian cancer, specifically to examine the susceptibility of the ovarian surface epithelium (OSE) and the tubal epithelial cells (TEC) in order to further clarify the origin of serous ovarian cancer. An *in vitro* model that examines the ovarian-specific microenvironmental could provide significant benefit to the field in the following areas: a system which induces transformation, without the need for a genetically engineered mouse; a transformation assay which utilizes normal primary cells, rather than genetically immortalized or cancer cells; and a prevention and drug responsiveness assay for future therapies. We propose to create such an *in vitro* model, in order to directly compare the susceptibility of OSE and TEC to specific genetic alterations in pathways associated with serous cancer formation and determine what aspects might encourage transformation in a cell type specific manner.

Figure 1. Expression of lentivirus encoding for PTEN and efficacy demonstrated in 2D and in 3D ovarian organ cultures.



BODY: We have made significant progress and achieved the major deliverables for year 1 of our proposed research. As outlined in our statement of work, our research plan for the first year was to generate viral shRNA and overexpression constructs for the transduction of mouse ovarian surface epithelium (MOSE) and mouse (fallopian/oviductal) tubal epithelial cells (MTEC). The viruses were to be tested for their ability to modify gene expression. During our first year of funding, we acquired and engineered the following viruses for our research plan: PTEN shRNA, p53 shRNA, BRCA1/2 shRNA, Rb shRNA and a negative scrambled shRNA control virus that also encoded for GFP. Plasmids were purchased, prepared, packaged into lentiviral particles utilizing HEK293 cells. The lentiviruses were then concentrated and used in a series of experiments to test their function *in vitro* in MOSE and MTEC cell lines and *in vivo* in 3D cultured ovaries and oviducts (**Figure 1**). While the 3D infection of the ovarian surface was highly effective and generated knockdown of PTEN or expression of the GFP reporter (**Figure 1 and 3**), the infection of TEC inside the oviductal lumen was inefficient and did not significantly alter PTEN or GFP expression. In

Figure 2. Lenti and adenoviruses could not efficiently transduce oviductal epithelium in 3D cultures.

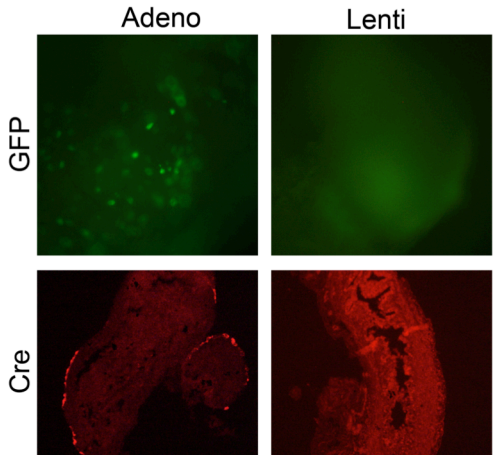


Figure 3. Floxed mice infected with virus encoding for GFP and cre-recombinase in vivo. Tubal cells are **rarely** infected and did not provide robust expression of GFP or cre-recombinase.

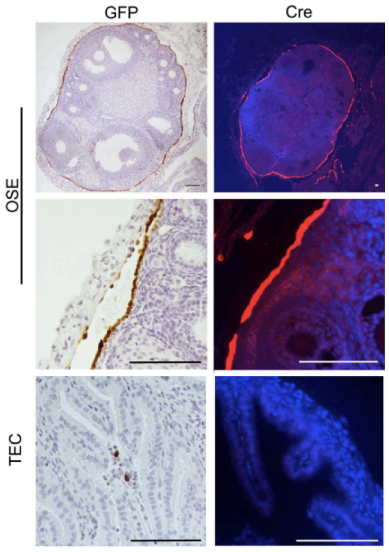
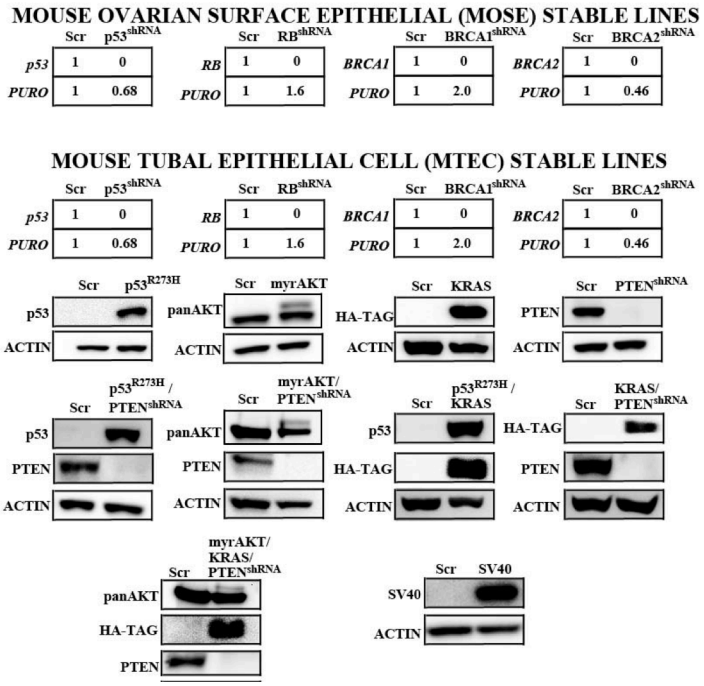
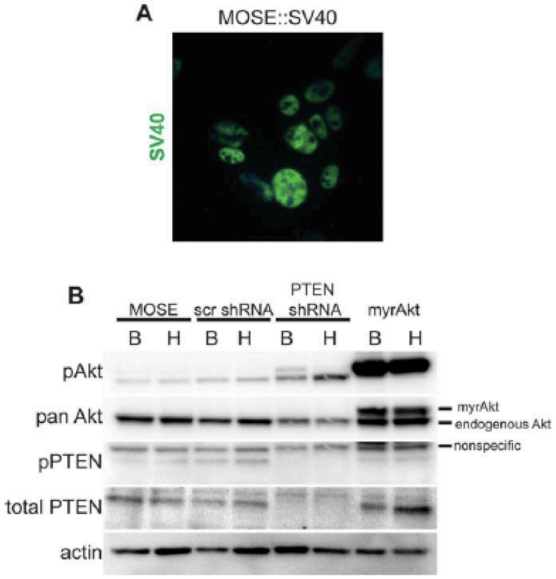


Figure 4. Stable cell lines generated with primary ovarian surface and oviductal epithelial cells.



order to examine this phenomenon further, mouse oviducts were isolated and both lentivirus and adenovirus encoding for GFP were injected inside the lumen of the oviduct, cultured overnight and monitored for GFP expression. GFP expression was not achieved with either virus type in TEC inside 3D cultured oviducts, an endpoint that is necessary for comparison to human disease initiation (**Figure 2**). As suggested in the pitfalls and alternative directions of our proposal, we purchased the transgenic animals that encode floxed alleles of interest (such as p53 deletion and mutant p53) to infect with virus as proof-of-principle that MTEC cells can be effectively transduced in 3D. These luminal oviductal cells *in vitro* and *in vivo* also did not infect readily with lentivirus or adenovirus (**Figure 3**). Therefore, as mentioned as another alternative direction, we generated normal primary mouse tubal epithelial cells (MTECs). We then engineered all of the stable cell lines suggested in our original proposal, as well as a few additional lines. These stable single, double, and triple transgenic MTEC lines are shown in **Figure 4**. We similarly engineered normal primary MOSE cell lines so that we could directly compare the susceptibility of the OSE to the TEC in terms of transformation

Figure 5. Primary MOSE stably expressing shRNA for PTEN, myr-Akt, and SV40T. Cells were treated with basal media (B) or hydrogen peroxide (H) to activate pAkt.



from specific pathway modifications as was the overall purpose of our application (**Figure 4 and 5**).

Since the time of our funding, transgenic animal models have been published derived from the mouse OSE using Rb deletion, p53 deletion, mutant p53, and BRCA1 and BRCA2 deletion alone and in combination, thus these pathways have already been confirmed to generate tumors when occurring simultaneously in the OSE[1]. In addition, Pten deletion with KRAS was published and reported to form low grade serous tumors from mouse OSE[2]. However, all publications on fallopian tube cells have used hTert and SV40 for immortalization in culture, and Hras or Myc to drive tumorogenesis[3,4]. The use of SV40 inactivates p53 and Rb, thus making the study of mutant p53 not feasible. To date, no publications describe the role of PTEN, Akt, Rb, mutant p53, p53 shRNA, or BRCA1/2 shRNA in oviductal cells as singly occurring alterations. Therefore, we focused our attention on these alterations in an MTEC model, as their characterization is necessary to understand how specific pathways modify and/or transform TEC, and to identify if these changes are similar to those seen in OSE and serous cancer. In addition, the findings from the Cancer Genome Atlas Network indicate that BRAF mutation is only associated with low grade serous cancer; therefore, it was eliminated from the list and replaced with myristoylated Akt to mimic activation of Akt, which is associated with high grade serous cancer in women[5].

Figure 6. MOSE cells with PTEN shRNA and myrAkt proliferate more rapidly than controls.

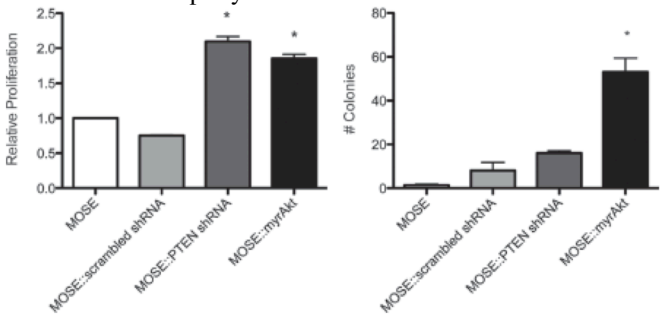
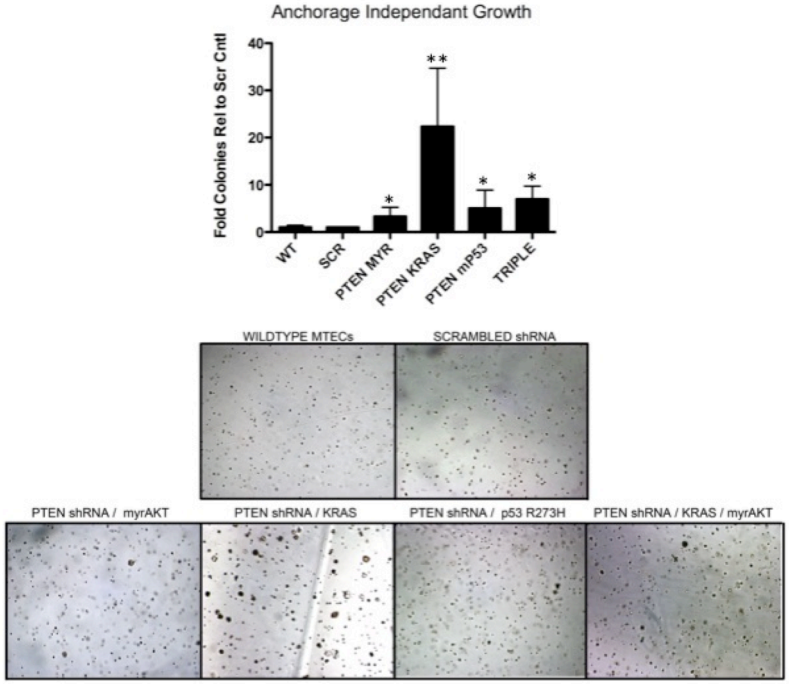


Figure 7. MTEC stable cell lines that demonstrated the ability to grow in soft agar.

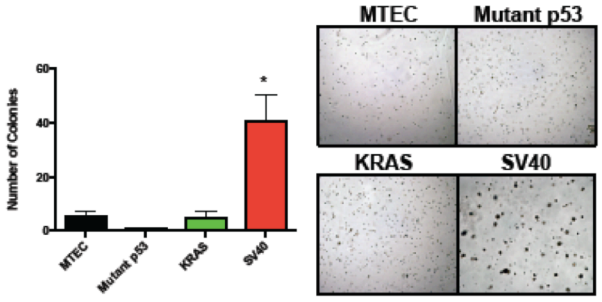


Our second major task, as stated in the SOW, was to assay the transformative potential of the cells after targeted knockdown or overexpression. These endpoints included assays for hyperplasia, invasion, p53 signatures, and changes in apoptosis. First, we investigated soft agar colony formation from all of the cells. We rationalized that if the cells do not form colonies, they are highly unlikely to form tumors. We prioritized cell models that would form tumors for all of the follow up analysis

of proliferation, migration, and apoptosis. However, given the ubiquitous nature of p53 mutation in HGSC, we analyzed mutant p53 cells in all assays.

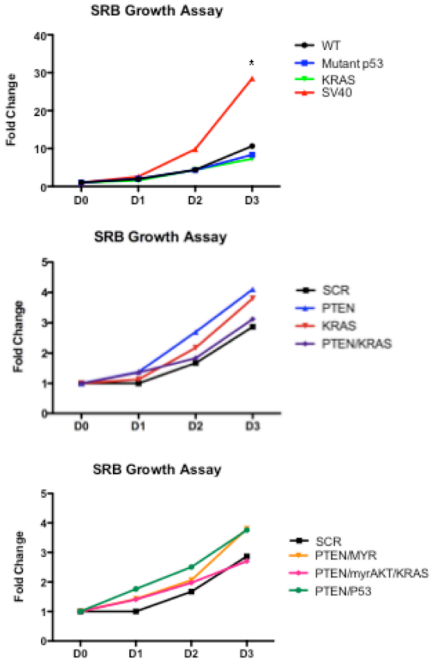
As shown in **Figure 6**, MOSE cells with myr-Akt and SV40 were able to form soft agar colonies. All other proposed combinations of genetic alterations in OSE have since been published to form serous or endometrioid cancer using intrabursal injections of virus that effectively transduces expression in OSE but not TEC [1,2,6,7]. Thus, our major focus has been to characterize the MTEC cells, because to date there are no tissue specific promoters for the fallopian tube and genetic alteration has not been introduced into primary cells that have functional p53. Engineering of otherwise normal MTEC cells demonstrated that PTEN shRNA/myrAkt, PTEN/KRAS(G12V), PTEN/mutant p53, and PTEN/KRAS/myrAkt can stimulate soft agar colony formation (**Figure 7**). The data for mutant p53 (R273H) alone, KRAS alone, and SV40 are provided (**Figure 8**) demonstrating single mutations alone are not sufficient for transformation. However, p53 is the most commonly mutated gene in serous cancer and is also mutated in early lesions of the fallopian tube. Therefore, we are further characterizing the downstream signaling of mutant p53, to further identify its role in early tubal cell derived serous tumor biology. In addition, in human cells, SV40 alone did not form soft agar colonies, but with the addition of Hras, tumors formed in nude mice[3,4]. We chose to study KRAS as its mutation is more common in serous tumors[5]. Neither p53 alone or KRAS alone stimulated soft agar colonies, while SV40 did. The SV40 result confirms *in vivo* studies indicating that murine expression of SV40 induces tumors[8].

Figure 8. Mutation in p53 or KRAS activation is not sufficient to stimulate soft agar colonies.



To examine how genetic alteration of MTEC cells effects proliferation and hyperplasia we have monitored the growth of our engineered cell models as compared to wild-type and scrambled shRNA control cells. We utilized SV40T as a positive control in MOSE and MTEC cells as this viral protein *in vivo* induces tumor formation[8,9]. While SV40 significantly increased proliferation, mutation in p53 (R273H) and KRAS (G12V) did not enhance proliferation of MTEC cells as compared to control (**Figure 9 top panel**). Next PTEN shRNA, KRASG12V, and PTEN/KRAS were compared to the scrambled control (**Figure 9 middle panel**). Lastly, PTEN/myrAkt, PTEN/myrAkt/KRAS and mutant PTEN/p53 were completed (**Figure 9 bottom panel**). Intriguingly, other then SV40, none of these single, double, and triple modifiers significantly

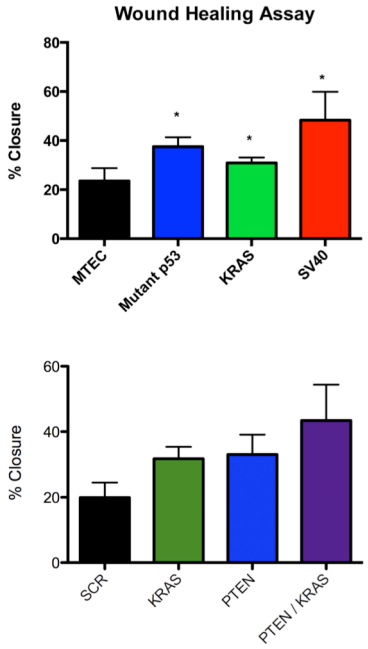
Figure 9. Proliferation for MTEC cell models.



impacted proliferation.

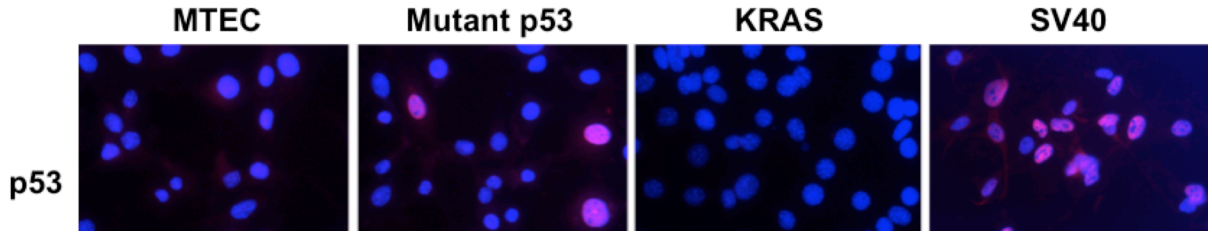
In order to monitor alterations in invasion with genetic alteration we have measured wound closure. MTEC cells expressing mutant p53, KRAS, and SV40T all increased wound closure as compared to wild-type cells (**Figure 10 top panel**). PTEN/KRAS, PTEN, and KRAS also appear to trend towards enhancing invasion, however additional experiments are required to determine if these trends are statistically significant. We are currently in the process of finalizing these experiments, as well as performing wound closures assays for the additional models that formed colonies in soft agar.

Figure 10. Would healing assays reveal enhanced migration of stable MTEC cell models.



In order to monitor formation of p53 signatures, we measured stabilization of p53 via western blot analysis and via immunocytochemistry. MTEC cells with mutant p53, KRAS and SV40 were stained for p53 to determine if the protein was stabilized similar to immunohistochemical staining performed in fallopian tubes of women at risk for serous cancer. We identified that mutation in p53 alone could enhance staining (**Figure 11**).

Figure 11. Mutant p53 cells demonstrate enhanced staining for p53 indicating protein stabilization.



Our research for the next year of funding will focus on assessing the histology, tumors, and/or metastases generated from nude mice xenografted with our genetically engineered MOSE and MTEC cell lines, which mirrors the original SOW for year 2. We anticipate that the cellular models that formed colonies in soft agar will be most likely to form tumors, and we will graft these models into mice first. As we have only recently generated MTEC cells with mutant p53/KRAS combined, we will assay them for proliferation, migration, soft agar growth, and tumor formation.

KEY RESEARCH ACCOMPLISHMENTS:

- All models were generated and validated.
- Single pathway modifications, including mutant p53 alone, did not induce transformation of tubal epithelial cells or ovarian surface epithelial cells.
- Mutant p53 and PTEN knockdown induced soft agar colonies.

- KRAS expression and PTEN knockdown induced soft agar colonies and increased tubal epithelial cell migration.
- Akt expression and PTEN knockdown induced soft agar colonies in MTEC cells.
- Akt/Pten/KRAS induced soft agar colonies in MTEC cells.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- **Abstracts and presentations**

Eddie, S.L., Burdette, J.E. Selective knockdown of PTEN in ovarian and oviductal epithelium is not sufficient to initiate cancerous transformation. Illinois Symposium for Reproductive Science. (poster)

Corona, P., Eddie, S.L., Burdette, J.E. Role of PTEN in fallopian tube and ovarian epithelium in initiation of serous cancer. UIC Research Symposium. (poster).

Eddie, S.L., Quartuccio, S.M., Shepherd, J.A., Kothari, R., and Burdette, J.E. Molecular signaling in the human fallopian tube: insights into proliferation and p53 expression. AACR Advances in Ovarian Cancer Research: From Concept to Clinic. Miami, FL (poster).

Eddie, S.L., Quartuccio, S.M., Shepherd, J.A., Kothari, R., and Burdette, J.E. Molecular signaling in the human fallopian tube: insights into proliferation and p53 expression. 5th Annual Illinois Symposium on Reproductive Biology. Carbondale, IL (oral-received second place prize).

Quartuccio, S.M., Eddie, S.L., O'hAinmhire, E., Talon, B., and Burdette, J.E. Downstream signaling from mutant p53 expression in normal oviductal tubal epithelium: Insights into serous cancer formation. 5th Annual Illinois Symposium on Reproductive Biology. Carbondale, IL (poster-received third place prize).

- **degrees obtained that are supported by this award**

Suzanne Quartuccio has an anticipated graduation date of May 15, 2014. She is currently preparing a manuscript and finalizing her dissertation.

- **development of cell lines, tissue or serum repositories**

All of the cell lines in **Figures 4** and **5** are new variants (14 MTEC stable cell lines and 6 MOSE).

- **funding applied for based on work supported by this award**

An RO3 and a DOD Pilot grant were submitted in June/August to support development of the human 3D fimbria culture.

CONCLUSION: Our results confirm that single alterations in pathways associated with high-grade serous cancer are not sufficient to drive soft agar colony formation as an index of transformation, but that specific combinations such as KRAS/Pten and mutant p53/Pten, which represent some of the most common pathways activated in human high grade serous cancer, are able to promote early events in tumor formation. Mutation of p53 in the OSE or in the TEC is not sufficient to drive tumorogenesis; however, mutation of p53 combined with PTEN deletion enhances migration and soft agar colony formation. Similarly, PTEN silencing combined with KRAS mutation can enhance oviductal migration and enhance growth in soft agar. These data help support mounting evidence that oviductal epithelium can be a source of serous cancer and provide excellent models for future studies that are aimed at uncovering signaling events downstream of mutant p53, Pten, KRAS, Rb, BRCA, and Akt. Furthermore, signal transduction downstream of common events, such as mutation in p53, that are not able to induced tumor formation alone can be studied to determine how they contribute to tumor formation. For example, our data indicates that Aurora kinase A and B are directly upregulated by mutation in p53, which could be one of the underlying factors contributing to chromosomal abnormalities seen in high grade serous cancer.

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APPENDICES: N/A